Kongeriget Danmark

Patent application No.: 1096/96

Date of filing: 04 Oct 1996

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TAASTRUP 09 Aug 1997

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Kontorfuldmægtig

NOVEL PROBES FOR THE DETECTION OF MYCOBACTERIA OF THE MYCOBACTERIUM TUBERCULOSIS COMPLEX

The present invention relates to the design, construction and use of novel probes for detecting mycobacteria of the Mycobacterium tuberculosis Complex (MTC) which probes are capable of detecting the organisms in test samples, e.g. expectorates, sputum, aspirates, urine, blood and tissue sections, food, soil and water.

BACKGROUND OF THE INVENTION

Tuberculosis caused by mycobacterial infection is presently the predominant infectious cause of morbidity and mortality world-wide, and is estimated to kill about three million people annually. WHO estimates that the annual number of new cases of tuberculosis will increase from 7.5 million in 1990 to 10.2 million in 2000, an escalation that will result in approximately 90 million new cases during this decade. It is furthermore estimated that 30 million people will die from tuberculosis during the 1990s, which equals one quarter of preventable deaths among adults.

The prevalence of tuberculosis has been very high in the poorer parts of the world such as Asia, Africa and South-America, but in recent years an increase has also been observed in industrialised countries. This appears to be due to an interaction of various factors including i.a. patterns of migration, poorly organised tuberculosis programmes and nutrition problems. Furthermore, a serious threat will arise from the emergence of new strains that are multi-drug resistant.

Considering the perspective and impact the disease has, the development of rapid, specific and preferably easy-performed and economic feasible diagnostic detection tests are of utmost importance and would be a very valuable tool in the fight against the spread of tuberculosis.

Generally, mycobacterial infections are divided into infections caused by two groups of bacteria, namely mycobacteria of the Mycobacterium tuberculosis Complex (MTC) and mycobacteria of the Mycobacterium avium-intracellulare Complex (MAC). The mycobacteria of the Mycobacterium tuberculosis Complex include M. tuberculosis, M. bovis and M. africanum, whereas the mycobaceria of the Mycobacterium avium-intracellulare Complex comprise M. avium and M. intracellulare.

Presently, the detection of mycobacteria by microscopy gives the more accurate diagnosis. The sample (e.g. an expectorate) is stained for acid-fast bacillus using Ziehl-Neelsen staining

and may subsequently be cultured in order to confirm the result obtained by staining. Such techniques are one of the cornerstones of all anti-tuberculosis programmes. However, the Ziehl-Neelsen staining lacks sensitivity since the detection limit is 10^4 organisms/ml or greater. On the contrary, cultivation is sensitive, and it may be possible to detect 10-100 organisms per sample, but the result is not available before up to 8 weeks of cultivation. Likewise, information of drug susceptibility is not available until after 2-3 weeks of further testing.

Automated detection is rapidly becoming available for large scale testing for the presence of mycobacteria. Such systems include ESP Myco Culture System (Difco), MB/BacT (Organon Teknika) and MGIT (Becton Dickinson). These test methods are based on colorimetric or fluorometric detection of carbon dioxide or oxygen produced by mycobacterial metabolism.

Neither staining nor cultivation methods allows distinction between the mycobacteria of the MTC and the MAC.

Some of the attempts to replace the methods based on cultivation rely on target amplification or target hybridisation using specific probes.

One of such newly developed target amplification method is based on PCR. The principle of this reaction is, through amplification of specific nucleic acid sequences of the mycobacteria, to increase the copy number of the specific sequence to a level where it may be detectable in an early stage of the infection. In principle, the PCR reaction offers the possibility of detecting as few as one target sequence. In most cases, the DNA is extracted prior to carrying out the PCR reaction. However, it has become clear that the method used to extract DNA from specimens has a great influence on the sensitivity and specificity of PCR products.

Furthermore, false negative results in specimens may be obtained due to the presence of inhibitors of the PCR reaction such as haemoglobin and proteins.

Another problem arises from cross-contamination of negative specimens with a bacteria not present in the sample. This may cause problems in conventional bacteriological procedures and may lead to a positive PCR result. Contamination of reagents and specimens with amplified PCR products is yet another well-recognised problem when using a PCR-based diagnosis.

Nucleic acid probes for detecting rRNA of mycobacteria have been described in for example US 5 547 842, EP-A 0 572 120 and US 5 422 242.

SUMMARY OF THE INVENTION

The present invention discloses and claims novel peptide nucleic acid probes for the detection of mycobacteria of the Mycobacterium tuberculosis Complex. The probes detect sequences in 16S rRNA and genomic sequences corresponding to said rRNA. rRNA is present in a high number of copies in each cell, and hence a well suited target for a sensitive test. Furthermore, probes that are complementary to rRNA are especially suitable for hybridisation as it is known that species variable regions exist within these highly conserved sequences thereby enabling the design of probes for detecting mycobacteria of the Mycobacterium tuberculosis Complex.

The novel probes may be used in an assay for the detection of mycobacteria of the MTC. The mycobacteria of the MTC are responsible for significant morbidity and mortality in humans. M. tuberculosis is the most common mycobacteria of the MTC isolated from humans. M. bovis may be transmitted from infected animals to humans. M. africanum causes pulmonary tuberculosis in tropical Africa.

Tuberculosis is highly contagious, and a rapid diagnosis of the disease is therefore very important. For most clinical laboratories, assignment of an isolate to the group of MTC bacteria is sufficient.

Thus, in a first aspect, the invention features a hybridisation assay probe able to detect mycobacteria of the MTC. Specifically, the probe is a peptide nucleic acid as defined in claim 1. Such probe sequences do not to any significant degree cross react with nucleic acid from other organisms in the test sample under appropriate stringency conditions.

In another aspect, the present invention relates to a method according to claim 7 for detecting the presence of organisms belonging to the group of mycobacteria of the MTC.

In yet another aspect, the present invention relates to a kit comprising at least one peptide nucleic acid probe as defined in anyone of claims 1 to 6.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows positions 1 to 200 of an alignment of 16S rRNA of M. intracellulare (positions 20 to 216 of GenBank entry GB:MIN16SRN, accession number x52927), M. avium (positions 30 to 227of GenBank entry GB:MAV16SRN, accession number x52918), B. bovis (positions 169 to 368 of GenBank entry MSGTGDA, accession number m20940) and M. tuberculosis (positions 30 to 229 of GenBank entry GB:MTU16SRNA, accession number x52917),

respectively.

Figure 2 shows positions 361 to 440, 1041 to 1080, 1161 to 1280 of an alignment of 16S rRNA of M. intracellulare (positions 377 to 456, 1057 to 1096, and 1177 to 1296 of GenBank entry GB:MIN16SRN, accession number x52927), M. avium (positions 388 to 467, 1068 to 1107, and 1188 to 1307 GenBank entry GB:MAV16SRN, accession number x52918), B. bovis (positions 529 to 608, 1209 to 1248, and 1329 to 1447 of GenBank entry MSGTGDA, accession number m20940) and M. tuberculosis (positions 390 to 469, 1070 to 1109, and 1190 to 1308 of GenBank entry GB:MTU16SRNA, accession number x52917), respectively.

SPECIFIC DESCRIPTION

The present invention provides novel probes for use in rapid and sensitive hybridisation based assays for the detection of organisms belonging to the group of mycobacteria of the MTC.

We have identified suitable variable regions of the target nucleic acid by comparative analysis of general available 16S rRNA sequences. Computers and computer programs which have been used for the purposes herein disclosed are generally available. In the probe design, sequence variations between the organisms belonging to the group of mycobacteria of the MTC and other organisms have been taken into consideration, in particular M. avium and M. intracellulare.

When designing the probes, due regard should be taken to the assay conditions under which the probes are to be used. The stringency of the assay conditions determines the degree of complementarity needed between the probe and nucleic acid forming a hybrid. Stringency is chosen so as to maximise the difference in stability between the hybrid formed with the target nucleic acid and the non-target nucleic acid. It is desirable to have probes which hybridise under conditions of high stringency. Under such conditions, only highly complementary nucleic acids will form hybrids with the probe according to the invention; hybrids without a sufficient degree of complementarity will not be formed.

Furthermore, probes should be positioned so as to minimise the stability of the probe:non-target nucleic acid hybrid. This may be accomplished by minimising the degree of complementarity to non-target nucleic acid and by designing the probe to span as many destabilising mismatches as possible. Whether a probe is useful to detect an organism belonging to the MTC group depends largely on the thermal stability difference between probe:target hybrids and probe:non-target hybrids. In designing the probes, the differences in these T_m values should be as large as possible.

Hybrids formed between peptide nucleic acid probes and nucleic acids have a higher thermal instability of mismatching bases compared to nucleic acid duplexes of the same sequences. Thus, the peptide nucleic acid probes exhibit a greater specificity for a complementary nucleic acid sequence than the traditional nucleic acid probe, which is seen as a greater difference in T_m values for probe:target hybrids and probe:non-target hybrids.

The length of the probe sequence is also important. The optimal length of a probe comprising a particular site of differences in base composition, e.g. among homologous regions of mycobacteria 16S rRNA, is a compromise between the principle that longer probes ensure specificity and shorter probes ensure that the destabilising differences in base composition form a greater part of the probe.

Peptide nucleic acids can form duplexes in either orientation, but the antiparallel orientation form the most regular and stable duplex. Hence the antiparallel configuration is preferred for probe applications.

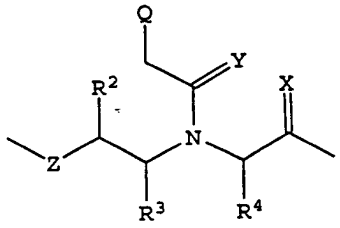
Mainly because the peptide nucleic acid strand is uncharged, a peptide nucleic acid-nucleic acid-duplex will have a higher T_m than the corresponding nucleic acid-nucleic acid-duplex. Typically there will be an increase in T_m of about 1 °C per basepair at 100 mM NaCl depending on the sequence (Egholm et al. (1993), Nature, 365, 566-568).

In contrast to DNA-DNA-duplex formation, no salt is necessary to facilitate and stabilise the formation of a peptide nucleic acid-DNA or a peptide nucleic acid-RNA duplex. The T_m of the peptide nucleic acid-DNA-duplex changes only a little with increasing ionic strength. Typically for a 15-mer, the T_m will drop only 5 °C when the salt concentration is raised from 10 mM NaCl to 1 M NaCl. At low ionic strength (e.g. 10 mM phosphate buffer with no salt added), it is possible to hybridise peptide nucleic acid to a target sequence under conditions where no stable DNA-DNA-duplex formation is able to occur (Nucleic Acid Hybridisation, a practical approach, eds. B. D. Hames & S. J. Higgins, IRL Press 1985, page 62-64). Furthermore, target sites that normally are inaccessible can be made more readily accessible for hybridisation with peptide nucleic acid probes at low salt concentration as the secondary and tertiary structure of nucleic acids are melted under such conditions.

Although it is preferred to use peptide nucleic acid probes targeting specific sequences of rRNA, it will readily be understood that peptide nucleic acid probes complementary to the rRNA targeting probes will be useful for the detection of the genes (DNA) coding for said sequence specific rRNA. Thus, as used herein, "probes able to form hybrids with target

sequences in 16S rRNA" refers to probes capable of hybridising to sequences in 16S rRNA or to corresponding sequences in the non-coding strand of the rDNA as well as it refers to complementary probes capable of hybridising to the coding strand of DNA coding for the target rRNA sequences.

In accordance with the present invention, peptide nucleic acid probes of formula (I) are provided, which probes are useful for detecting mycobacteria of the Mycobacterium tuberculosis Complex (MTC) in a sample, and which probes comprise from 10 to 30 polymerised moieties of formula (I)



(I)

wherein each X and Y independently designate O or S,
each Z independently designates O, S, NR^1, or $C(R^1)_2$, wherein each R^1 independently designate H, C_{1-6} alkyl, C_{1-6} alkenyl, C_{1-6} alkynyl,
each R^2, R^3 and R^4 designate independently H, the side chain of a naturally occurring amino acid, the side chain of a non-naturally occurring nucleobase, C_{1-4} alkyl, C_{1-4} alkenyl or C_{1-4} alkynyl, or a functional group, each Q independently designates a naturally occurring nucleobase, a non-naturally occurring nucleobase, an intercalator, a nucleobase-binding group, a label or H,

with the proviso that Q forms a sequence comprising a subsequence complementary to a target sequence in 16S rRNA of said mycobacteria, in which subsequence a nucleobase complementary to the nucleobase at at least one of the following positions is included

Position 9 in Figure 1, or
Position 20 in Figure 1, or
Positions 9 and 20 in Figure 1, or
Position 55-56 in Figure 1, or
Position 114 in Figure 1, or
Positions 114 and 116-117 in Figure 1, or
Position 120 in Figure 1, or
Positions 116-117 and 120 in Figure 1, or
Positions 114, 116-117 and 120 in Figure 1, or

Position 141-143 in Figure 1, or
Positions 120 and 141-143 in Figure 1, or
Position 161 in Figure 1, or
Positions 141-143 and 161 in Figure 1, or
Position 393 in Figure 2, or
Position 1055-1056 in Figure 2, or
Position 1063-1064 in Figure 2, or
Positions 1055-1056 and 1063-1064 in Figure 2, or
Position 1191 in Figure 2, or
Position 1206-1208 in Figure 2,
Positions 1191 and 1206-1208 in Figure 2, or
Position 1232 in Figure 2, or
Positions 1206-1208 and 1232 in Figure 2, or
Position 1253 in Figure 2, or
Positions 1232 and 1253 in Figure 2,

and further with the proviso that the probe comprising such subsequence is able to form hybrids with target sequences in 16S rRNA of said mycobacteria.

The term "naturally occurring nucleobases" includes the four main DNA bases (i.e. thymine (T), cytosine (C), adenine (A) and guanine (G)) as well as other naturally occurring nucleobases (e.g. uracil (U) and hypoxanthine).

The term "non-naturally occurring nucleobases" comprises i.a. modified naturally occurring nucleobases. Such non-naturally occurring nucleobases may be modified by substitution by e.g. one or more C_{1-8} alkyl, C_{1-8} alkenyl or C_{1-8} alkynyl groups or labels. Examples of non-naturally occurring nucleobases are purine, 2,6-diamino purine, 5-propynylcytosine (C propynyl), isocytosine (iso-C), 5-methyl-isocytosine ($iso^{Me}C$) (see e.g. Tetrahedron Letters Vol 36, No 12, 2033-2036 (1995) or Tetrahedron Letters Vol 36, No 21, 3601-3604 (1995)), 7-deazaadenine, 7-deazaguanine, N^4-ethanocytosine, N^6-ethano-2,6-diaminopurine, 5-(C_{3-6})-alkenyluracil, 5-(C_{3-6})-alkynylcytosine, 5-fluorouracil and pseudocytosine.

Examples of useful intercalators are e.g. acridin, antraquinone, psoralen and pyrene.

Examples of useful nucleobase-binding groups are e.g. groups containing cyclic or heterocyclic rings. Non-limiting examples are 3-nitro pyrrole and 5-nitro indole.

It is to be understood that alkyl, alkenyl and alkynyl groups may be branched or non-branched,

cyclic or non-cyclic, and may further be interrupted by one or more heteroatoms, or may be unsubtituted or substituted by or may contain one or more functional groups. Non-limiting examples of such functional groups are acetyl groups, acyl groups, amino groups, carbamido groups, carbamoyl groups, carbamyl groups, carbonyl groups, carboxy groups, cyano groups, dithio groups, formyl groups, guanidino groups, halogens, hydrazino groups, hydrazo groups, hydroxamino groups, hydroxy groups, keto groups, mercapto groups, nitro groups, phospho groups, phospho ester groups, sulfo groups, thiocyanato groups, cyclic, aromatic and heterocyclic groups.

C_{1-4} groups contain from 1 to 4 carbon atoms, C_{1-6} groups contain from 1 to 6 carbon atoms, and C_{1-15} groups contain from 1 to 15 carbon atoms, not including optional substituents, heteroatoms and/or functional groups.

Non-limiting examples of such groups are -OH, $-CH_3$, $-CF_3$, $-CH_2-$, $-CH_2CH_3$, $-CH_2CH_2-$, $-CH(CH_3)_2$, $-OCH_3$, $-OCH_2-$, $-OCH_2CH_3$, $-OCH_2CH_2-$, $-OCH(CH_3)_2$, $-OC(O)CH_3$, $-OC(O)CH_2-$, -C(O)H, -C(O)-, $-C(O)CH_3$, -C(O)OH, -C(O)O-, $-CH_2NH_2$, $-CH_2NH-$, $-CH_2OCH_3$, $-CH_2OCH_2-$, $-CH_2OC(O)OH$, $-CH_2OC(O)O-$, $-CH_2C(O)CH_3$, $-CH_2C(O)CH_2-$, $-C(O)NH_2$, $-P(O)_4H$, -SH, $-NH_2$, $-CH=CH_2$, -CH=CH-, $-CH=CHCH_2C(O)OH$, $-CH=CHCH_2C(O)O-$, $-C\equiv CH$, $-C\equiv C-$, $-CH_2C\equiv CH$, $-CH_2C\equiv C-$, $-CH_2C\equiv CCH_3$, $-OCH_2C\equiv CH$, $-OCH_2C\equiv C-$, $-OCH_2C\equiv CCH_3$, $-NHCH_2C(O)-$, $-NHCH_2CH_2C(O)-$, $-NH(CH_2CH_2O)_2CH_2C(O)-$, and $HO(O)CCH_2C(O)(NH-(CH_2CH_2O)_2CH_2C(O))_2-$, phenyl, benzyl, naphthyl, oxazolyl, pyridinyl, thiadiazolyl, triazolyl, and thienyl.

Within the present context, the expression "naturally occurring amino acid" is intended to comprise D- and L-forms of amino acids commonly found in nature, e.g. D- and L-forms of Ala (alanine), Arg (arginine), Asn (aspargine), Asp (aspartic acid), Cys (cysteine), Gln (glutamine), Glu (glutamic acid), His (histidine), Ile (isoleucine), Leu (leucine), Lys (lysine), Met (methionine), Phe (phenylalanine), Pro (proline), Ser (serine), Thr (threonine), Trp (tryptophan), Tyr (tyrosine) and Val (valine).

In the present context, the expression "non-naturally occurring amino acid" is intended to comprise D- and L-forms of amino acids other than those commonly found in nature as well as modified naturally occurring amino acids. Examples of useful non-naturally occurring amino acids are D- and L-forms of Cha (cyclohexylalanine), Cit (citrulline), Hci (homocitrulline), HomoCys (homocystein), Hse (homoserine), Nle (norleucine), Nva (norvaline), Orn (ornithine), Sar (sarcosine) and Thi (thienylalanine).

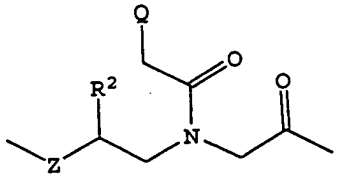
The strength of the binding between the probe and the nucleic acid sequence is further

influenced by the ligand Q. When Q designates a nucleobase, Hoogsteen and/or Watson-Crick base pairing assists in the formation of hybrids between a nucleic acid sequence to be detected and the probe. It is contemplated that one or more of the ligands may be a group which contribute little or none to the binding of the nucleic acid such as hydrogen. It is contemplated that suitable probes to be used comprise less than 25% by weight of moieties, wherein Q designates such groups. One or more of the ligands Q may be groups that stabilise nucleobase stacking such as intercalators or nucleobase-binding groups.

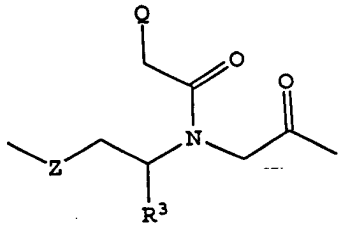
In the above-indicated probes one or more of the Q-groups may designate a label. Examples of suitable labels are given below. Moieties wherein Q denotes a label may preferably be located in one or both of the terminating moieties of the probe. Moieties wherein Q denotes a label may also be located internally.

The peptide nucleic acid probes may comprise moieties, wherein all X groups are O (polyamides) or wherein all X groups are S (polythioamides). It is to be understood that the probes may also comprise mixed moieties (comprising both amide and thioamides).

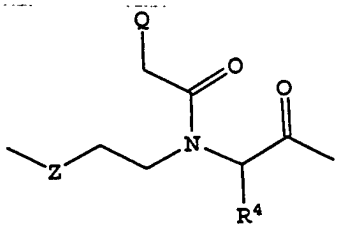
In another aspect, the present invention relates to peptide nucleic acid probes of formula (II), (III) and (IV)



(II)



(III)



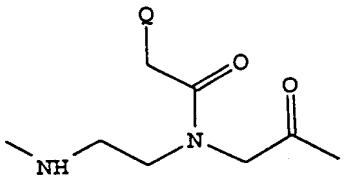
(IV)

wherein Z, R^2, R^3, and R^4, and Q is as defined above, which probes are suitable for detecting mycobacteria of the MTC.

In a preferred embodiment, the peptide nucleic acid probes according to the invention are of formulas (I)-(IV) as defined above with Z being NH, NCH_3 or O, each R^2, R^3 and R^4 independently being the side chain of a naturally occurring nucleobase, the side chain of a non-naturally occurring nucleobase, or C_{1-4} alkyl, and each Q being a naturally occurring nucleobase or a non-naturally occurring nucleobase.

Peptide nucleic acid probes according to the invention are preferably those of formula (I)-(IV) as defined above, wherein Z is NH or O, and R^2 is H or the side chain of Ala, Asp, Cys, Glu, His, HomoCys, Lys, Orn, Ser or Thr, and Q is a nucleobase selected from thymine, adenine, cytosine, guanine, uracil, iso-C, iso-G and 2,6-diaminopurine.

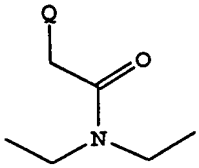
Peptide nucleic acid probes, which are of major interest for detecting mycobacteria of the MTC group, are probes of formula (V)



(V)

wherein R^4 is H or the side chain of Ala, Asp, Cys, Glu, His, HomoCys, Lys, Orn, Ser or Thr, and Q is as defined above and with the provisos above.

The peptide nucleic acid probe comprises polymerised moieties as defined above. From the formula, it is to be understood that the probe may comprise polymerised moieties which structure may be mutually different or identical. It may be advantageous that at least one moiety of the probe, preferably one (or both) of the moieties terminating the probe, are of a different structure. Such terminating moieties may suitably be a moiety of formula (VI)



(VI)

where Q is as defined above.

The preferred length of the probe will depend on the target material and whether labelled probes are used. It is contemplated that especially interesting probes comprise from 10 to 30 polymerised moieties as defined above. Probes of the invention may suitably comprise from 12 to 25 polymerised moieties, more suitably from 14 to 22 polymerised moieties, most suitably from 15 to 20 polymerised moieties.

As mentioned above, the polymerised moieties of the probes may be mutually different or identical. In some embodiments, the polymerised moieties of formulas (V) constitute at least 75% by weight (calculated by excluding labels and linkers), preferably at least 80% by weight and most preferably at least 90% by weight of the probe.

The ends on the moieties terminating the probe may be substituted by suitable substituents which in the following will be named "linkers". A terminating end may suitably be substituted by from 1 to 5 linkers, more suitably from 1 to 3 linkers. Such linkers may suitably be selected among C_{1-15} alkyl, C_{1-15} alkenyl and C_{1-15} alkynyl groups as defined above. The linkers may be substituted or unsubstituted, branched or non-branched, or be interrupted by heteroatoms, or be substituted or contain functional groups as described above. This may depend on the chemical nature of the terminating moiety (i.e. whether the moiety is terminated by a carbon, oxygen or nitrogen atom). A terminating end or a linker on a terminating end may further be substituted by one or more labels, which labels may be incorporated end to end, i.e. so as to form a non-branched labelled end, or may be incorporated so as to form a branched labelled end ("zipper"). The linkers may be attached directly to a terminating end, may be attached to a label or between labels on a terminating end, or be attached to a terminating end before a label is attached to a terminating end. It should be understood that two terminating ends may carry different or identical substituents, linkers and/or labels. It should further be understood that the term "a label" is intended to comprise one or more labels as the term "linkers" is to comprise one or more linkers.

Examples of suitable linkers are $-NH(CH_2CH_2O)_nCH_2C(O)-$, $-NH(CHOH)_nC(O)-$, $-(O)C(CH_2OCH_2)_nC(O)-$ and $-NH(CH_2)_nC(O)-$, $NH_2(CH_2CH_2O)_nCH_2C(O)-$, $NH_2(CHOH)_nC(O)-$, $HO(O)C(CH_2OCH_2)_nC(O)-$, $NH_2(CH_2)_nC(O)-$, $-NH(CH_2CH_2O)_nCH_2C(O)OH$, $-NH(CHOH)_nC(O)OH$, $-(O)C(CH_2OCH_2)_nC(O)OH$ and $-NH(CH_2)_nC(O)OH$, wherein n is 0 or an integer from 1 to 8, preferably from 1 to 3. Examples of very interesting linkers are $-NHCH_2C(O)-$, $-NHCH_2CH_2C(O)-$, $-NH(CH_2CH_2O)_2CH_2C(O)-$, $HO(O)CCH_2CH_2C(O)(NH-(CH_2CH_2O)_2CH_2C(O))_2-$.

In the present context, the term "label" refers to a substituent which is useful for detection or visualisation. Suitable labels comprise fluorophores, biotin, dinitro benzoic acid, digoxigenin, radioisotope labels, peptide or enzyme labels, chemiluminiscence labels, hapten, antigen or antibody labels.

The expression "peptide label" is intended to mean a label comprising from 1 to 20 naturally occurring or non-naturally occurring amino acids, preferably from 1 to 10 naturally occurring or

non-naturally occurring amino acids, more preferably from 1 to 8 naturally occurring or non-naturally occurring amino acids, most preferably from 1 to 4 naturally occurring or non-naturally occurring amino acids, linked together end to end in a non-branched or branched ("zipper") fashion. In a preferred embodiment, such a non-branched or branched end comprises one or more, preferably from 1 to 8 labels, more preferably from 1 to 4, further labels other than a peptide label. Such further labels may suitably terminate a non-branched end or a branched end. One or more linkers may suitably be attached to the terminating end before a peptide label and/or a further label is attached. Such linker units may also be attached between a peptide label and a further label.

The probe as such may also comprise one or more labels such as from 1 to 8, preferably from 1 to 4, labels and/or one or more linker units, which may be attached internally, i.e. to the backbone of the probe. The linker units and labels may mutually be attached as described above.

Examples of particular interesting labels are biotin, fluorescent labels, such as fluorescein labels, e.g. 5-(and 6)-carboxyfluorescein, 5- or 6-carboxyfluorescein, 6-(fluorescein)-5-(and 6)-carboxamido hexanoic acid and fluorescein isothiocyanate, peptide labels consisting of from 1 to 20 naturally occurring amino acids or non-naturally occurring amino acids, peroxidases such as horse radish peroxidase (HRP) and soya bean peroxidase, dinitro benzoic acid, rhodamine, tetramethylrhodamine, cyanine dyes such as Cy2, Cy3 and Cy5, coumarin, R-phycoerythrin (RPE), allophycoerythrin, Texas Red and Princeton Red as well as conjugates of R-phycoerythrin and, e.g. Cy5 or Texas Red.

Examples of preferred labels are biotin, fluorescent labels, peptide labels and dinitro benzoic acid. Peptide labels may preferably be composed of from 1 to 10, more preferably of from 1 to 8, most preferably of from 1 to 4, naturally occurring or non-naturally occurring amino acids. It may be particularly advantageous to incorporate one or more labels other as well as a peptide label such as from 1 to 8 or from 1 to 4 other labels.

Suitable peptide labels may preferably be composed of cysteine, glycine, lysine or ornithine.

In a further embodiment, the Q substituent as defined above may be labelled. Suitable labels are as defined above. Between Q and such a label, a linker as defined above may be incorporated. It is preferred that such labelled ligands Q are selected from thymine and uridine labelled in the 5-position.

The probes may be synthesised according to the procedures described in "PNA Information

Package" obtained from Millipore Corporation (Bedford, MA, USA), or may be synthesised on an Expedite Nucleic Acid Synthesis System (PerSeptive, USA).

If using the Fmoc strategy for elongation of the probe with linkers or amino acids, it was possible to retain side chain amino groups protected with acid sensitive protection groups such as the Boc group. This method allows introduction of a linker containing several Boc protected amino groups which can all be cleaved and labelled in the same synthesis cycle.

One way of labelling a probe is to use a fluorescent label, such as 5-(and 6)-carboxyfluorescein, 5- or 6-carboxyfluorescein, 6-(fluorescein)-5-(and 6)-carboxamido hexanoic acid or fluorescein isothiocyanate. The acid group is activated with HATU (O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) or HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and reacted with the N-terminal amino group. The same technique can be applied to other labelling groups containing an acid function. Alternatively, the succinimidyl ester of the above-mentioned labels may be used directly.

After synthesis, probes were cleaved from the resin using standard procedures as described by Millipore Corporation or PerSeptive Biosystems. The probes were purified and analysed using reversed-phase HPLC techniques at 50°C and were characterised by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOFMS), plasma desorption mass spectrometry (PDMS), electron spray mass spectrometry (ESMS), or fast atom bombardment (FAB-MS).

Generally, probes such as probes comprising polymerised moieties of formula (V) may also be prepared as described in, e.g., Tetrahedron Letters Vol 35, No 29, 5173-5176 (1994) and Bioorganic & Medical Chemistry Letters, Vol 4, No 8, 1077-1080 (1994). Chemical properties of some probes are described in, e.g., Nature, 365, 566-568 (1993).

Detection of the label depend on the type of label and on the format of the procedure. In cases where the sample is deposited onto slides, the hybridisation results may be visualised using well known immunohistochemical staining methods to detect the labelling on the probe. When fluorescent labelled binding partners are used, the hybrids may be detected using an antibody against the fluorescent label which antibody may be conjugated with an enzyme. The fluorescent label may alternatively be detected directly using a fluorescence microscope, or the results may be automatically analysed on a fluorescent-based image analysis system.

When biotin labelled probes are used, the hybrids may be detected using an antibody against the biotin label which antibody may be conjugated with an enzyme. If necessary, an

enhancement of the signal can be generated using commercially available amplification systems such as the catalysed signal amplification system for biotinylated probes (DAKO K 1500).

The probes according to the invention are used in the detection of mycobacteria of the MTC in samples which may contain these bacteria.

In the assay method, at least one probe according to the invention is contacted with target nucleic acid and an analysis for hybrid formation is carried out.

In the assay method according to the invention, a sample to be analysed for the presence of mycobacteria of the MTC is contacted with one or more probes according to the invention under such conditions by which hybridisation between the probe and any complementary sample rRNA of mycobacteria of the MTC can occur, and observing or measuring the resulting hybridisation.

In one embodiment of the assay method, conventionally prepared smears of bacterial cells are contacted with one or more probes according to the invention under conditions suitable for hybridisation to occur between the probe(s) and any complementary rRNA in the sample. The complexes formed are detected. An example of this assay format is fluorescence *in situ* hybridisation (FISH), wherein the probes according to the invention are labelled with fluorescein or another fluorophore. When designing MTC probes, it might be advantageous to use more than one probe. If e.g. three such probes are included in the assay each in a concentration of one third of the concentration of a single probe, possible cross reactivity of the individual probes will not invalidate the results.

In another embodiment of the assay method, a test sample is firstly subjected to conditions, which release nucleic acid from the bacteria present in that sample. Contact between one or more probes as defined herein, which may be labelled, and the rRNA target may be carried out in solution under conditions, which promote hybridisation between the probe(s) and any target nucleic acid present. The probe:nucleic acid complex may be immobilised to a solid support, e.g. by using a capture probe.

Due to the high affinity of the probes defined herein for nucleic acids, it is not necessary to carry out the hybridisation of the probe and nucleic acid in solution. This allows flexibility in the assay format. For instance, the detection probes can be brought into contact with the target nucleic acid in solution and the probe/nucleic acid complex can be captured by an immobilised capture probe. Or the sample comprising the target nucleic acid can even be added to an

assay system comprising detection probes as well as immobilised capture probe. The immobilisation of the capture probe may be effected by using a streptavidin coated solid phase and a biotinylated capture probe. The probe may be immobilised onto a solid support by coupling reaction between a carboxylic acid on the linker and an amino derivatised support. Alternatively, the coupling onto the solid support may be accomplished by photochemical activation of photoreactive groups which have been attached absorptively to the solid support prior to photochemical activation. Such photoreactive groups are described in EP 408 078 A.

In practice, a solid phase based assay system is very attractive as the analysis can be carried out using a solid phase precoated with a capture probe. A solid phase based assay system is also feasible for automatisation of the analysis.

The capture probe may be one of the other probes for detecting mycobacteria of the MTC not used in the hybridisation reaction and detection step for target nucleic acid, thus ensuring dual species specificity. The dual specificity will allow shorter probes be used, e.g. 10 mer probes.

The solid support capture system may take a wide variety of forms well known in the art, such as e.g. a plate, a microtiter plate having one or more wells, a microscope slide, a filter, a membrane, a tube, a dip stick, a strip, beads such as paramagnetic beads, beads made of polystyrene, polypropylene, polyethylene, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides and agaroses. When a filter, a membrane, a strip or beads is (are) used as the solid support, it (they) may, if conveniently be incorporated into a single-use.

It has been observed that peptide nucleic acids bind to a variety of solid phases. A blocking reaction is required to reduce non-specific binding of the peptide nucleic acids to the solid phase. The blocking reaction may be carried out with commonly used blocking reagents, such as BSA (bovine serum albumin), casein, Triton X-100® or Tween 20®. The preferred blocking reagents are Triton X-100® and Tween 20®.

The captured probe:nucleic acid complexes may be detected or identified by a wide variety of methods for that purpose. The probe to be brought in contact with the target nucleic acid may be labelled, whereby said may form part of the detection system. In another embodiment, the captures probe:nucleic acid complexes are detected using a detection system based on an antibody reacting specifically with complexes formed between peptide nucleic acid and nucleic acid (such as described in WO 95/17430), in which detection system the primary antibody may comprise a label, or which detection system comprises a labelled secondary antibody, which specifically binds to the primary antibody.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Examples of suitable sequences of Q are given below. Peptide nucleic acid probes comprising such sequences of Q will be able to detect mycobacteria of the MTC group.The probes are written from left to right corresponding to from the C-terminal end towards the N-terminal end.

The peptide nucleic acid probes shown below includes nucleobases complementary to Position 9 i Figure 1.

CTC CGA AGA GAC CTT TCC G
TC CGA AGA GAC CTT TCC G
C CGA AGA GAC CTT TCC G
CGA AGA GAC CTT TCC G
GA AGA GAC CTT TCC G
A AGA GAC CTT TCC G
AGA GAC CTT TCC G
GA GAC CTT TCC G
A GAC CTT TCC G
GAC CTT TCC G
CTC CGA AGA GAC CTT TCC
CTC CGA AGA GAC CTT TC
CTC CGA AGA GAC CTT T
CTC CGA AGA GAC CTT
CTC CGA AGA GAC CT
CTC CGA AGA GAC C
CTC CGA AGA GAC
CTC CGA AGA GA
TC CGA AGA GAC CTT TCC
C CGA AGA GAC CTT TC
CGA AGA GAC CTT T
GA AGA GAC CTT

Peptide nucleic acid probes including nucleobases complementary to Position 20 are e.g.

GTT CGC CAC TCG AGT ATC TCC GAA GAG
TT CGC CAC TCG AGT ATC TCC GAA GAG
T CGC CAC TCG AGT ATC TCC GAA GAG
CGC CAC TCG AGT ATC TCC GAA GAG
GC CAC TCG AGT ATC TCC GAA GAG
C CAC TCG AGT ATC TCC GAA GAG

CAC TCG AGT ATC TCC GAA GAG

AC TCG AGT ATC TCC GAA GAG

C TCG AGT ATC TCC GAA GAG

TCG AGT ATC TCC GAA GAG

CG AGT ATC TCC GAA GAG

GTT CGC CAC TCG AGT ATC TCC GAA GA

GTT CGC CAC TCG AGT ATC TCC GAA G

GTT CGC CAC TCG AGT ATC TCC GAA

GTT CGC CAC TCG AGT ATC TCC GA

GTT CGC CAC TCG AGT ATC TCC G

GTT CGC CAC TCG AGT ATC TCC

GTT CGC CAC TCG AGT ATC TC

GTT CGC CAC TCG AGT ATC T

GTT CGC CAC TCG AGT ATC

TT CGC CAC TCG AGT ATC TCC GAA GA

T CGC CAC TCG AGT ATC TCC GAA G

CGC CAC TCG AGT ATC TCC GAA

GC CAC TCG AGT ATC TCC GA

C CAC TCG AGT ATC TCC G

CAC TCG AGT ATC TCC

The peptide nucleic acid probes shown below includes nucleobases complementary to Positions 9 and 20 in Figure 1.

CAC TCG AGT ATC TCC GAA GAG **A**CC TTT CCG

AC TCG AGT ATC TCC GAA GAG **A**CC TTT CCG

C TCG AGT ATC TCC GAA GAG **A**CC TTT CCG

TCG AGT ATC TCC GAA GAG **A**CC TTT CCG

CG AGT ATC TCC GAA GAG **A**CC TTT CCG

G AGT ATC TCC GAA GAG **A**CC TTT CCG

AGT ATC TCC GAA GAG **A**CC TTT CCG

GT ATC TCC GAA GAG **A**CC TTT CCG

T ATC TCC GAA GAG **A**CC TTT CCG

ATC TCC GAA GAG **A**CC TTT CCG

TC TCC GAA GAG **A**CC TTT CCG

CAC TCG AGT ATC TCC GAA GAG **A**CC TTT CC

CAC TCG AGT ATC TCC GAA GAG **A**CC TTT C

CAC TCG AGT ATC TCC GAA GAG **A**CC TTT

CAC TCG AGT ATC TCC GAA GAG **A**CC TT

CAC TCG AGT ATC TCC GAA GAG **A**CC T
CAC TCG AGT ATC TCC GAA GAG **A**CC
CAC TCG AGT ATC TCC GAA GAG **A**C
CAC TCG AGT ATC TCC GAA GAG **A**
AC TCG AGT ATC TCC GAA GAG **A**CC TTT CC
C TCG AGT ATC TCC GAA GAG **A**CC TTT C
TCG AGT ATC TCC GAA GAG **A**CC TTT
CG AGT ATC TCC GAA GAG **A**CC TT
G AGT ATC TCC GAA GAG **A**CC T
AGT ATC TCC GAA GAG **A**CC
GT ATC TCC GAA GAG **A**C
T ATC TCC GAA GAG **A**

The following peptide nucleic acid probes includes nucleobases complementary to Position 55-56 i Figure 1.

CGA AGT GCA GGG CAG AT**C** **A**CC CAC GTG TTA
GA AGT GCA GGG CAG AT**C** **A**CC CAC GTG TTA
A AGT GCA GGG CAG AT**C** **A**CC CAC GTG TTA
AGT GCA GGG CAG AT**C** **A**CC CAC GTG TTA
GT GCA GGG CAG AT**C** **A**CC CAC GTG TTA
T GCA GGG CAG AT**C** **A**CC CAC GTG TTA
GCA GGG CAG AT**C** **A**CC CAC GTG TTA
CA GGG CAG AT**C** **A**CC CAC GTG TTA
A GGG CAG AT**C** **A**CC CAC GTG TTA
GGG CAG AT**C** **A**CC CAC GTG TTA
GG CAG AT**C** **A**CC CAC GTG TTA
G CAG AT**C** **A**CC CAC GTG TTA
CAG AT**C** **A**CC CAC GTG TTA
AG AT**C** **A**CC CAC GTG TTA
G AT**C** **A**CC CAC GTG TTA
AT**C** **A**CC CAC GTG TTA
T**C** **A**CC CAC GTG TTA
C **A**CC CAC GTG TTA
CGA AGT GCA GGG CAG AT**C** **A**CC CAC GTG TT
CGA AGT GCA GGG CAG AT**C** **A**CC CAC GTG T
CGA AGT GCA GGG CAG AT**C** **A**CC CAC GTG
CGA AGT GCA GGG CAG AT**C** **A**CC CAC GT
CGA AGT GCA GGG CAG AT**C** **A**CC CAC G

CGA AGT GCA GGG CAG ATC ACC CAC
CGA AGT GCA GGG CAG ATC ACC CA
CGA AGT GCA GGG CAG ATC ACC C
CGA AGT GCA GGG CAG ATC ACC
CGA AGT GCA GGG CAG ATC AC
CGA AGT GCA GGG CAG ATC A
GA AGT GCA GGG CAG ATC ACC CAC GTG TT
A AGT GCA GGG CAG ATC ACC CAC GTG T
AGT GCA GGG CAG ATC ACC CAC GTG
GT GCA GGG CAG ATC ACC CAC GT
T GCA GGG CAG ATC ACC CAC G
GCA GGG CAG ATC ACC CAC
CA GGG CAG ATC ACC CA
A GGG CAG ATC ACC C
GGG CAG ATC ACC

The following peptide nucleic acid probes are examples of probes including nucleobases complementary to Position 114 in Figure 1.

GTGG TCC TAT CCG GTA TTA GAC CCA
GTGG TCC TAT CCG GTA TTA GAC CC
GTGG TCC TAT CCG GTA TTA GAC C
GTGG TCC TAT CCG GTA TTA GAC
GTGG TCC TAT CCG GTA TTA GA
GTGG TCC TAT CCG GTA TTA G
GTGG TCC TAT CCG GTA TTA
GTGG TCC TAT CCG GTA TT
GTGG TCC TAT CCG GTA T
GTGG TCC TAT CCG GTA
GTGG TCC TAT CCG GT
GTGG TCC TAT CCG G
GTGG TCC TAT CCG
GTGG TCC TAT CC
GTGG TCC TAT C

The peptide nucleic acid probes indicated below includes nucleobases complementary to Positions 114 and 116-117 in Figure 1.

TCC CGT GGT CCT ATC CGG TA
CC CGT GGT CCT ATC CGG TA

C CGT GGT CCT ATC CGG TA
TCC CGT GGT CCT ATC CGG T
TCC CGT GGT CCT ATC CGG T
TCC CGT GGT CCT ATC CGG
TCC CGT GGT CCT ATC CG
TCC CGT GGT CCT ATC C
TCC CGT GGT CCT ATC
TCC CGT GGT CCT AT
TCC CGT GGT CCT A
TCC CGT GGT CCT
TCC CGT GGT CC
TCC CGT GGT C
CC CGT GGT CCT ATC CGG T
C CGT GGT CCT ATC CGG

The peptide nucleic acid probes indicated below includes nucleobases complementary to Positions 114, 116-117 and 120 in Figure 1.

CAC AAG ACA TGC ATC CCG TGG TCC TAT CCG
AC AAG ACA TGC ATC CCG TGG TCC TAT CCG
C AAG ACA TGC ATC CCG TGG TCC TAT CCG
AAG ACA TGC ATC CCG TGG TCC TAT CCG
AG ACA TGC ATC CCG TGG TCC TAT CCG
G ACA TGC ATC CCG TGG TCC TAT CCG
ACA TGC ATC CCG TGG TCC TAT CCG
CA TGC ATC CCG TGG TCC TAT CCG
A TGC ATC CCG TGG TCC TAT CCG
TGC ATC CCG TGG TCC TAT CCG
GC ATC CCG TGG TCC TAT CCG
C ATC CCG TGG TCC TAT CCG
ATC CCG TGG TCC TAT CCG
CAC AAG ACA TGC ATC CCG TGG TCC TAT CC
CAC AAG ACA TGC ATC CCG TGG TCC TAT C
CAC AAG ACA TGC ATC CCG TGG TCC TAT
CAC AAG ACA TGC ATC CCG TGG TCC TA
CAC AAG ACA TGC ATC CCG TGG TCC T
CAC AAG ACA TGC ATC CCG TGG TCC
CAC AAG ACA TGC ATC CCG TGG TC
CAC AAG ACA TGC ATC CCG TGG T

CAC AAG ACA TGC **A**TC **CC**G **T**GG
CAC AAG ACA TGC **A**TC **CC**G **T**G
CAC AAG ACA TGC **A**TC **CC**G **T**
AC AAG ACA TGC **A**TC **CC**G **T**GG TCC TAT CC
C AAG ACA TGC **A**TC **CC**G **T**GG TCC TAT C
AAG ACA TGC **A**TC **CC**G **T**GG TCC TAT
AG ACA TGC **A**TC **CC**G **T**GG TCC TA
G ACA TGC **A**TC **CC**G **T**GG TCC T
ACA TGC **A**TC **CC**G **T**GG TCC
CA TGC **A**TC **CC**G **T**GG TC
A TGC **A**TC **CC**G **T**GG T
TGC **A**TC **CC**G **T**GG
GC **A**TC **CC**G **T**G
GC **A**TC **CC**G **T**GG TCC T

The following peptide nucleic acid probes are examples of probes including nucleobases complementary to Position 120 in Figure 1.

TCC ACC ACA AGA CAT GC**A** TC
CC ACC ACA AGA CAT GC**A** TC
C ACC ACA AGA CAT GC**A** TC
ACC ACA AGA CAT GC**A** TC
CC ACA AGA CAT GC**A** TC
C ACA AGA CAT GC**A** TC
ACA AGA CAT GC**A** TC
CA AGA CAT GC**A** TC
A AGA CAT GC**A** TC
AGA CAT GC**A** TC
GA CAT GC**A** TC
TCC ACC ACA AGA CAT GC**A** T
TCC ACC ACA AGA CAT GC**A**
CC ACC ACA AGA CAT GC**A** T
C ACC ACA AGA CAT GC**A**

As examples of peptide nucleic acid probes including nucleobases complementary to Position 141-143 in Figure 1, the following probes are given.

CCG CTA AA**G** **CG**C TTT CCA C
CG CTA AA**G** **CG**C TTT CCA C
G CTA AA**G** **CG**C TTT CCA C

CTA AA**G CG**C TTT CCA C
TA AA**G CG**C TTT CCA C
A AA**G CG**C TTT CCA C
AA**G CG**C TTT CCA C
A**G CG**C TTT CCA C
G CGC TTT CCA C
CCG CTA AA**G CG**C TTT CCA
CCG CTA AA**G CG**C TTT CC
CCG CTA AA**G CG**C TTT C
CCG CTA AA**G CG**C TTT
CCG CTA AA**G CG**C TT
CCG CTA AA**G CG**C T
CCG CTA AA**G CG**C
CCG CTA AA**G CG**
CG CTA AA**G CG**C TTT CCA
G CTA AA**G CG**C TTT CC
CTA AA**G CG**C TTT C
TA AA**G CG**C TTT

The following peptide nucleic acid probes are examples of probes including nucleobases complementary to Positions 120 and 141-143 in Figure 1.

GCT AAA **GCG** CTT TCC ACC ACA AGA CAT GC**A**
CT AAA **GCG** CTT TCC ACC ACA AGA CAT GC**A**
T AAA **GCG** CTT TCC ACC ACA AGA CAT GC**A**
AAA **GCG** CTT TCC ACC ACA AGA CAT GC**A**
AA **GCG** CTT TCC ACC ACA AGA CAT GC**A**
A **GCG** CTT TCC ACC ACA AGA CAT GC**A**
GCG CTT TCC ACC ACA AGA CAT GC**A**

Examples of peptide nucleic acid probes including nucleobases complementary to Position 161 in Figure 1 are the following.

GCT GAT AGG CCG CGG GC**T** CAT CCC ACA CCG
CT GAT AGG CCG CGG GC**T** CAT CCC ACA CCG
T GAT AGG CCG CGG GC**T** CAT CCC ACA CCG
GAT AGG CCG CGG GC**T** CAT CCC ACA CCG
AT AGG CCG CGG GC**T** CAT CCC ACA CCG
T AGG CCG CGG GC**T** CAT CCC ACA CCG
AGG CCG CGG GC**T** CAT CCC ACA CCG

GG CCG CGG GCT CAT CCC ACA CCG
G CCG CGG GCT CAT CCC ACA CCG
CCG CGG GCT CAT CCC ACA CCG
CG CGG GCT CAT CCC ACA CCG
G CGG GCT CAT CCC ACA CCG
CGG GCT CAT CCC ACA CCG
GG GCT CAT CCC ACA CCG
G GCT CAT CCC ACA CCG
GCT CAT CCC ACA CCG
CT CAT CCC ACA CCG
T CAT CCC ACA CCG
GCT GAT AGG CCG CGG GCT CAT CCC ACA CC
GCT GAT AGG CCG CGG GCT CAT CCC ACA C
GCT GAT AGG CCG CGG GCT CAT CCC ACA
GCT GAT AGG CCG CGG GCT CAT CCC AC
GCT GAT AGG CCG CGG GCT CAT CCC A
GCT GAT AGG CCG CGG GCT CAT CCC
GCT GAT AGG CCG CGG GCT CAT CC
GCT GAT AGG CCG CGG GCT CAT C
GCT GAT AGG CCG CGG GCT CAT
GCT GAT AGG CCG CGG GCT CA
GCT GAT AGG CCG CGG GCT C
GCT GAT AGG CCG CGG GCT
CT GAT AGG CCG CGG GCT CAT CCC ACA CC
T GAT AGG CCG CGG GCT CAT CCC ACA C
GAT AGG CCG CGG GCT CAT CCC ACA
AT AGG CCG CGG GCT CAT CCC AC
T AGG CCG CGG GCT CAT CCC
AGG CCG CGG GCT CAT CC
GG CCG CGG GCT CAT C
G CCG CGG GCT CAT
CCG CGG GCT CA

Examples of peptide nucleic acid probes including Positions 141-143 and 161 are

GCC GCG GGC TCA TCC CAC ACC GCT AAA **GCG**
CC GCG GGC TCA TCC CAC ACC GCT AAA **GCG**
C GCG GGC TCA TCC CAC ACC GCT AAA **GCG**
GCG GGC TCA TCC CAC ACC GCT AAA **GCG**

CG GGC **T**CA TCC CAC ACC GCT AAA **GCG**
G GGC **T**CA TCC CAC ACC GCT AAA **GCG**
GGC **T**CA TCC CAC ACC GCT AAA **GCG**
GC **T**CA TCC CAC ACC GCT AAA **GCG**
C **T**CA TCC CAC ACC GCT AAA **GCG**
TCA TCC CAC ACC GCT AAA **GCG**

Peptide nucleic acid probes directed against Position 393 in Figure 2 are for instance the following.

CCA CCT ACC GTC AAT CCG AGA **G**AA CCC GGA
CA CCT ACC GTC AAT CCG AGA **G**AA CCC GGA
A CCT ACC GTC AAT CCG AGA **G**AA CCC GGA
CCT ACC GTC AAT CCG AGA **G**AA CCC GGA
CT ACC GTC AAT CCG AGA **G**AA CCC GGA
T ACC GTC AAT CCG AGA **G**AA CCC GGA
ACC GTC AAT CCG AGA **G**AA CCC GGA
CC GTC AAT CCG AGA **G**AA CCC GGA
C GTC AAT CCG AGA **G**AA CCC GGA
GTC AAT CCG AGA **G**AA CCC GGA
TC AAT CCG AGA **G**AA CCC GGA
C AAT CCG AGA **G**AA CCC GGA
AAT CCG AGA **G**AA CCC GGA
AT CCG AGA **G**AA CCC GGA
T CCG AGA **G**AA CCC GGA
CCG AGA **G**AA CCC GGA
CG AGA **G**AA CCC GGA
G AGA **G**AA CCC GGA
AGA **G**AA CCC GGA
GA **G**AA CCC GGA
A **G**AA CCC GGA
CCA CCT ACC GTC AAT CCG AGA **G**AA CCC GG
CCA CCT ACC GTC AAT CCG AGA **G**AA CCC G
CCA CCT ACC GTC AAT CCG AGA **G**AA CCC
CCA CCT ACC GTC AAT CCG AGA **G**AA CC
CCA CCT ACC GTC AAT CCG AGA **G**AA C
CCA CCT ACC GTC AAT CCG AGA **G**AA
CCA CCT ACC GTC AAT CCG AGA **G**A
CCA CCT ACC GTC AAT CCG AGA **G**

CA CCT ACC GTC AAT CCG AGA **G**AA CCC GG
A CCT ACC GTC AAT CCG AGA **G**AA CCC G
CCT ACC GTC AAT CCG AGA **G**AA CCC
CT ACC GTC AAT CCG AGA **G**AA CC
T ACC GTC AAT CCG AGA **G**AA C
ACC GTC AAT CCG AGA **G**AA
CC GTC AAT CCG AGA **G**A
C GTC AAT CCG AGA **G**

Peptide nucleic acid probes including nucleobases complementary to Position 1055-1056 in Figure 2 are for instance the following.

CAT TAC **GT**G CTG GCA ACA TGA
AT TAC **GT**G CTG GCA ACA TGA
T TAC **GT**G CTG GCA ACA TGA
TAC **GT**G CTG GCA ACA TGA
AC **GT**G CTG GCA ACA TGA
C **GT**G CTG GCA ACA TGA
GTG CTG GCA ACA TGA
CAT TAC **GT**G CTG GCA ACA TGA
CAT TAC **GT**G CTG GCA ACA TG
CAT TAC **GT**G CTG GCA ACA T
CAT TAC **GT**G CTG GCA ACA
CAT TAC **GT**G CTG GCA AC
CAT TAC **GT**G CTG GCA A
CAT TAC **GT**G CTG GCA
CAT TAC **GT**G CTG GC
CAT TAC **GT**G CTG G
CAT TAC **GT**G CTG
CAT TAC **GT**G CT
CAT TAC **GT**G C
T TAC **GT**G CTG GCA AC
TAC **GT**G CTG GCA A
AC **GT**G CTG GCA

Peptide nucleic acid probes including nucleobases complementary to Position 1063-1064 in Figure 2 are for instance the following.

T TCT CTC ACG AGT CCC C**AC** CAT TAC
TCT CTC ACG AGT CCC C**AC** CAT TAC

CT CTC ACG AGT CCC C**AC** CAT TAC
T CTC ACG AGT CCC C**AC** CAT TAC
CTC ACG AGT CCC C**AC** CAT TAC
TC ACG AGT CCC C**AC** CAT TAC
C ACG AGT CCC C**AC** CAT TAC
ACG AGT CCC C**AC** CAT TAC
CG AGT CCC C**AC** CAT TAC
G AGT CCC C**AC** CAT TAC
AGT CCC C**AC** CAT TAC
GT CCC C**AC** CAT TAC
T CCC C**AC** CAT TAC
CCC C**AC** CAT TAC
CC C**AC** CAT TAC
C C**AC** CAT TAC
TCT CTC ACG AGT CCC C**AC** CAT TA
CT CTC ACG AGT CCC C**AC** CAT T
T CTC ACG AGT CCC C**AC** CAT
CTC ACG AGT CCC C**AC** CA
TC ACG AGT CCC C**AC** C
C ACG AGT CCC C**AC**
ACG AGT CCC C**AC** CAT TA
CG AGT CCC C**AC** CAT T
G AGT CCC C**AC** CAT
AGT CCC C**AC** CA

Peptide nucleic acid probes including nucleobases complementary to Positions 1055-1056 and 1063-1064 in Figure 2 are for instance the following.

GTC CCC **AC**C ATT AC**G** **T**GC TGG CAA
TC CCC **AC**C ATT AC**G** **T**GC TGG CAA
C CCC **AC**C ATT AC**G** **T**GC TGG CAA
CCC **AC**C ATT AC**G** **T**GC TGG CAA
CC **AC**C ATT AC**G** **T**GC TGG CAA
C **AC**C ATT AC**G** **T**GC TGG CAA
ACC ATT AC**G** **T**GC TGG CAA
GTC CCC **AC**C ATT AC**G** **T**GC TGG CA
GTC CCC **AC**C ATT AC**G** **T**GC TGG C
GTC CCC **AC**C ATT AC**G** **T**GC TGG
GTC CCC **AC**C ATT AC**G** **T**GC TG

GTC CCC **ACC** ATT AC**G** **T**GC T
GTC CCC **ACC** ATT AC**G** **T**GC
GTC CCC **ACC** ATT AC**G** **T**G
GTC CCC **ACC** ATT AC**G** **T**
TC CCC **ACC** ATT AC**G** **T**GC TGG CA
C CCC **ACC** ATT AC**G** **T**GC TGG C
CCC **ACC** ATT AC**G** **T**GC TGG
CC **ACC** ATT AC**G** **T**GC TG
C **ACC** ATT AC**G** **T**GC T
ACC ATT AC**G** **T**GC

Peptide nucleic acid probes including nucleobases complementary to Position 1191 in Figure 2 are e.g.

CTT AAC CT**C** GCG GCA TCG
TT AAC CT**C** GCG GCA TCG
T AAC CT**C** GCG GCA TCG
AAC CT**C** GCG GCA TCG
AC CT**C** GCG GCA TCG
C CT**C** GCG GCA TCG
CT**C** GCG GCA TCG
T**C** GCG GCA TCG
C GCG GCA TCG
CTT AAC CT**C** GCG GCA TC
CTT AAC CT**C** GCG GCA T
CTT AAC CT**C** GCG GCA
CTT AAC CT**C** GCG GC
CTT AAC CT**C** GCG G
CTT AAC CT**C** GCG
CTT AAC CT**C** GC
CTT AAC CT**C** G
TT AAC CT**C** GCG GCA TC
T AAC CT**C** GCG GCA T
AAC CT**C** GCG GCA

Examples of peptide nucleic acid probes including nucleobases complementary to Position 1206-1208 are e.g.

GGC TTT TA**A** **G**GA TTC GCT
GC TTT TA**A** **G**GA TTC GCT

C TTT TA**A** **G**GA TTC GCT
TTT TA**A** **G**GA TTC GCT
TT TA**A** **G**GA TTC GCT
T TA**A** **G**GA TTC GCT
TA**A** **G**GA TTC GCT
A**A** **G**GA TTC GCT
A **G**GA TTC GCT
GGC TTT TA**A** **G**GA TTC GC
GGC TTT TA**A** **G**GA TTC G
GGC TTT TA**A** **G**GA TTC
GGC TTT TA**A** **G**GA TT
GGC TTT TA**A** **G**GA T
GGC TTT TA**A** **G**GA
GGC TTT TA**A** **G**G
GGC TTT TA**A** **G**
GC TTT TA**A** **G**GA TTC GC
C TTT TA**A** **G**GA TTC G
TTT TA**A** **G**GA TTC
TT TA**A** **G**GA TT

Examples of peptide nucleic acid probes including nucleobases complementary to Positions 1191 and 1206-1208 in Figure 2 are e.g.

CTT TTA **AG**G ATT CGC TTA ACC T**C**G CGG CAT
TT TTA **AG**G ATT CGC TTA ACC T**C**G CGG CAT
T TTA **AG**G ATT CGC TTA ACC T**C**G CGG CAT
TTA **AG**G ATT CGC TTA ACC T**C**G CGG CAT
TA **AG**G ATT CGC TTA ACC T**C**G CGG CAT
A **AG**G ATT CGC TTA ACC T**C**G CGG CAT
CTT TTA **AG**G ATT CGC TTA ACC T**C**G CGG CA
CTT TTA **AG**G ATT CGC TTA ACC T**C**G CGG C
CTT TTA **AG**G ATT CGC TTA ACC T**C**G CGG
CTT TTA **AG**G ATT CGC TTA ACC T**C**G CG
CTT TTA **AG**G ATT CGC TTA ACC T**C**G C
CTT TTA **AG**G ATT CGC TTA ACC T**C**G
CTT TTA **AG**G ATT CGC TTA ACC T**C**
TT TTA **AG**G ATT CGC TTA ACC T**C**G CGG CA
T TTA **AG**G ATT CGC TTA ACC T**C**G CGG C
TTA **AG**G ATT CGC TTA ACC T**C**G CGG

TA **AG**G ATT CGC TTA ACC T**C**G CG
A **AG**G ATT CGC TTA ACC T**C**G C
AGG ATT CGC TTA ACC T**C**G

Examples of peptide nucleic acid probes including nucleobases complementary to Position 1232 in Figure 2 are e.g.
CAG ACC CC**G** ATC CGA AC
AG ACC CC**G** ATC CGA AC
G ACC CC**G** ATC CGA AC
ACC CC**G** ATC CGA AC
CC CC**G** ATC CGA AC
C CC**G** ATC CGA AC
CC**G** ATC CGA AC
C**G** ATC CGA AC
CAG ACC CC**G** ATC CGA A
CAG ACC CC**G** ATC CGA
CAG ACC CC**G** ATC CG
CAG ACC CC**G** ATC C
CAG ACC CC**G** ATC
CAG ACC CC**G** AT
CAG ACC CC**G** A
AG ACC CC**G** ATC CGA A
G ACC CC**G** ATC CGA
ACC CC**G** ATC CG

Examples of peptide nucleic acid probes including nucleobases complementary to Positions 1206-1208 and 1232 in Figure 2 are
CC**G** ATC CGA ACT GAG ACC GGC TTT TA**A** **G**GA
C**G** ATC CGA ACT GAG ACC GGC TTT TA**A** **G**GA
G ATC CGA ACT GAG ACC GGC TTT TA**A** **G**GA
CC**G** ATC CGA ACT GAG ACC GGC TTT TA**A** **G**G
CC**G** ATC CGA ACT GAG ACC GGC TTT TA**A** **G**

Examples of peptide nucleic acid probes including nucleobases complementary to Position 1253 in Figure 2 are
CCG ACT TCA **C**GG GGT CGA G
CG ACT TCA **C**GG GGT CGA G
G ACT TCA **C**GG GGT CGA G

ACT TCA **C**GG GGT CGA G
CT TCA **C**GG GGT CGA G
T TCA **C**GG GGT CGA G
TCA **C**GG GGT CGA G
CA **C**GG GGT CGA G
A **C**GG GGT CGA G
CGG GGT CGA G
CCG ACT TCA **C**GG GGT CGA
CCG ACT TCA **C**GG GGT CG
CCG ACT TCA **C**GG GGT C
CCG ACT TCA **C**GG GGT
CCG ACT TCA **C**GG GG
CCG ACT TCA **C**GG G
CCG ACT TCA **C**GG
CCG ACT TCA **C**G
CCG ACT TCA **C**
CG ACT TCA **C**GG GGT CG
G ACT TCA **C**GG GGT C
ACT TCA **C**GG GGT
CT TCA **C**GG GG

Examples of peptide nucleic acid probes including nucleobases complementary to Positions 1232 and 1253 in Figure 2 are

TTC A**C**G GGG TCG AGT TGC AGA CCC C**G**A TCC
TC A**C**G GGG TCG AGT TGC AGA CCC C**G**A TCC
C A**C**G GGG TCG AGT TGC AGA CCC C**G**A TCC
A**C**G GGG TCG AGT TGC AGA CCC C**G**A TCC
CG GGG TCG AGT TGC AGA CCC C**G**A TCC
TTC A**C**G GGG TCG AGT TGC AGA CCC C**G**A TC
TTC A**C**G GGG TCG AGT TGC AGA CCC C**G**A T
TTC A**C**G GGG TCG AGT TGC AGA CCC C**G**A
TTC A**C**G GGG TCG AGT TGC AGA CCC C**G**
TC A**C**G GGG TCG AGT TGC AGA CCC C**G**A TC
C A**C**G GGG TCG AGT TGC AGA CCC C**G**A T
A**C**G GGG TCG AGT TGC AGA CCC C**G**A
CG GGG TCG AGT TGC AGA CCC C**G**

EXAMPLES

EXAMPLE 1

In situ hybridisation to fixed bacterial cells

To test the ability of the peptide nucleic acid probes to detect MTC and not MAC or Neisseria gonorrhoeae, fluorescence *in situ* hybridisation (FISH) was performed on fixed bacterial cells using fluorescein labelled probes as shown below. It was shown that these probes did not hybridise to M. avium, M. intracellulare, or N. gonorrhoeae.

Preparation of bacterial slides

M. bovis BCG (Statens Seruminstitut, Denmark, Catalogue number 2645), M. avium (Statens Seruminstitut, Denmark, Laboratory number 3716 (E37978)), and M. intracellulare (Statens Seruminstitut, Laboratory number 3717 (E39562)) were grown in Dubos medium (Statens Seruminstitut, Denmark) or on Löwenstein-Jensen medium (Statens Seruminstitut, Denmark) at 37 °C. N. gonorrhoeae was grown on chocolate agar at 37 °C with additional 5% CO_2.

Bacterial smears were prepared on test slides according to standard procedures. The smears were air-dried followed by flame fixation.

FISH on bacterial slides

The following procedure was performed.

1. The slide is immersed in 80% ethanol for 15 minutes, subsequently rinsed with water and air-dried.
2. The bacterial slide is covered with a hybridisation solution containing the probe in question at a concentration of 250 nM.
3. The slide is incubated in a humid incubation chamber at 45 °C for 90 minutes.
4. The slide is washed 25 minutes in TBS-buffer, pH 10 at 45 °C, followed by 30 seconds in water.
5. The slide is dried and mounted (DAKO Fluorescence Mounting Medium or equivalent).

The following hybridisation solutions was used:

Hybridisation solution	10 mM NaCl 10% Dextran sulphate 30% formamide 0.1% Triton X-100® 50 mM Tris-HCl, pH 7.6 50 mM EDTA

0.1% sodium pyrophosphate
0.2% polyvinylpyrrolidone
0.2% Ficol

TBS buffer 10 mM sodium phosphate, pH 10
145 mM NaCl

All solutions are made RNase free following standard procedures.

The following peptide nucleic acid probe was used

Lys(Flu)-Lys(Flu)-CAC AGG ACA TGC ATC-NH_2 OK 310

wherein Flu denotes a fluorescein isothiocyanate label or a 5-(and 6)-carboxyfluorescein label, and Lys(Flu)-Lys(Flu) denotes a peptide label ("zipper") with two Flu labels attached. The results are shown in Table 1.

TABLE 1

Probe OK 310	FISH
M. bovis BCG	positive
M. avium	negative
M. intracellulare	negative
N. gonorrhoeae	negative

EXAMPLE 2

Test in dot blots

To further test the ability of the peptide nucleic acid probes to detect MTC and not MAC or E. coli, dot blot tests were carried out.

M. bovis BCG (Statens Seruminstitut Catalogue number 2645) and M. intracellulare (Statens Seruminstitut, Denmark Laboratory number 3713 (E39562)) were grown in Dubos medium (Statens Seruminstitut, Denmark) or on Löwenstein-Jensen medium (Statens Seruminstitut, Denmark) at 37 °C.

RNA was isolated from the bacterial cells by use of TRI-reagent (Sigma) following manufacture's directions. E. coli rRNA was purchased from Boehringer Mannheim, Germany.

The following nucleic acid probes were used.

Lys(Flu)-Lys(Flu)-Gly-GCA TCC CGT GGT CCT-NH_2	OK 223
Lys(Flu)-Lys(Flu)-CAC AGG ACA TGC ATC-NH_2	OK 310

wherein Flu denotes a fluorescein isothiocyanate label or a 5-(and 6)-carboxyfluorescein label, and Lys(Flu)-Lys(Flu)-Gly and Lys(Flu)-Lys(Flu) denote peptide labels ("zippers") consisting of 3 and 2 amino acids, respectively, with two Flu labels attached.

Preparation of dot blots

The following buffers were used:

20 × SSPE buffer	3 M NaCl
	0.2 M PO_4^{3-}
	0.02 M EDTA
	pH 7.4
TST buffer	0.05 M Tris/HCl
	0.5 M NaCl
	0.5% Tween 20®
	pH 9.0

200 ng M. bovis RNA, M. intracellulare RNA and E. coli rRNA were dotted onto membranes (Schleich & Schuel, NY 13 N), and the membranes were dried and fixed under UV light for 2 minutes. Each of the probes (70 nM probe in hybridisation solution (hybridisation solution without Triton X-100® and with the exception that formamide was substituted with 50% glycerol)) were added to the membrane. Hybridisation was continued for 1.5 hours at 55 °C. The membranes were rinsed 2 times for 15 minutes in 2 × SSPE buffer containing 0.1% SDS at ambient temperature, and subsequently 2 times for 15 minutes in 0.1 × SSPE buffer containing 0.1% SDS at 55 °C or at 65 °C (see Table 2). The membrane was blocked with 0.5% casein dissolved in 0.05M Tris/HCl and 0.5 M NaCl with pH 9.0. Thereafter, the membranes were incubated for 1 hour with rabbit-anti FITC antibody labelled with AP (DAKO K0046 vial A) diluted 1:2000 in 0.5% casein dissolved in 0.05M Tris/HCl and 0.5 M NaCl with pH 9.0. After incubation, the membranes were washed 3 times 5 minutes with TST at ambient temperature. Bound probes were visualised following standard procedures using BCIP/NBT,

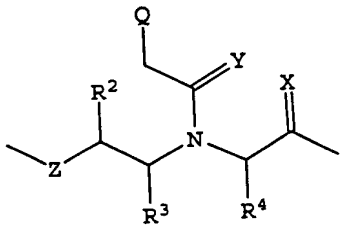
and the visualisation was stopped by incubation for 10 minutes with 10 mM EDTA. The blot was dried at 50 °C.

The results are given in Table 2 below. The term "nd" denotes "not determined".

	E. coli rRNA		M. bovis BCG RNA		M. intracellulare RNA	
Probe	55 °C	65 °C	55 °C	65 °C	55 °C	65 °C
OK 223	negative	negative	positive	positive	nd	nd
OK 310	negative	negative	negative	positive	negative	negative

CLAIMS

1. A peptide nucleic acid probe for detecting mycobacteria of the Mycobacterium tuberculosis Complex (MTC) in a sample, which probe comprises from 10 to 30 polymerised moieties of formula (I)



(I)

wherein each X and Y independently designate O or S,
each Z independently designates O, S, NR^1, or $C(R^1)_2$, wherein each R^1 independently designate H, C_{1-6} alkyl, C_{1-6} alkenyl, C_{1-6} alkynyl,
each R^2, R^3 and R^4 designate independently H, the side chain of a naturally occurring amino acid, the side chain of a non-naturally occurring nucleobase, C_{1-4} alkyl, C_{1-4} alkenyl or C_{1-4} alkynyl, or a functional group, each Q independently designates a naturally occurring nucleobase, a non-naturally occurring nucleobase, an intercalator, a nucleobase-binding group, a label or H,

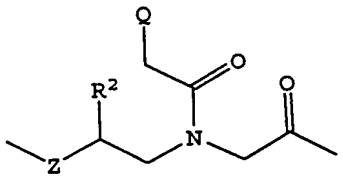
with the proviso that Q forms a sequence comprising a subsequence complementary to a target sequence in 16S rRNA of said mycobacteria, in which subsequence a nucleobase complementary to the nucleobase at at least one of the following positions is included

Position 9 in Figure 1, or
Position 20 in Figure 1, or
Positions 9 and 20 in Figure 1, or
Position 55-56 in Figure 1, or
Position 114 in Figure 1, or
Positions 114 and 116-117 in Figure 1, or
Position 120 in Figure 1, or
Positions 116-117 and 120 in Figure 1, or
Positions 114, 116-117 and 120 in Figure 1, or
Position 141-143 in Figure 1, or
Positions 120 and 141-143 in Figure 1, or
Position 161 in Figure 1, or
Positions 141-143 and 161 in Figure 1, or

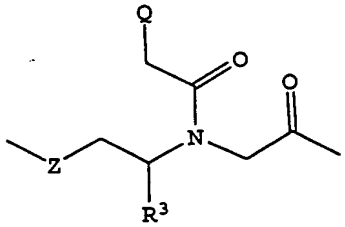
Position 393 in Figure 2, or
Position 1055-1056 in Figure 2, or
Position 1063-1064 in Figure 2, or
Positions 1055-1056 and 1063-1064 in Figure 2, or
Position 1191 in Figure 2, or
Position 1206-1208 in Figure 2,
Positions 1191 and 1206-1208 in Figure 2, or
Position 1232 in Figure 2, or
Positions 1206-1208 and 1232 in Figure 2, or
Position 1253 in Figure 2, or
Positions 1232 and 1253 in Figure 2,

and further with the proviso that the probe comprising such subsequence is able to form hybrids with target sequences in 16S rRNA of said mycobacteria.

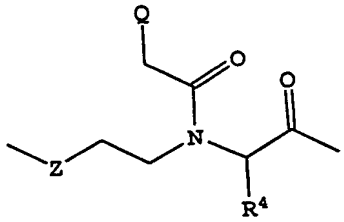
2. A peptide nucleic acid probe according to claim 1 of formula (II), (III), or (IV)



(II)



(III)



(IV)

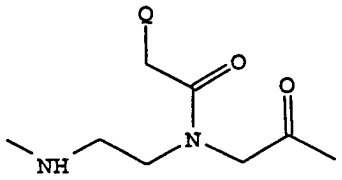
wherein Z, R^2, R^3, and R^4, and Q is as defined in claim 1.

3. A peptide nucleic acid probe according to claim 1 or 2, wherein Z is NH, NCH_3 or O, each R^2, R^3 and R^4 independently designate the side chain of a naturally occurring nucleobase, the side chain of a non-naturally occurring nucleobase, or C_{1-4} alkyl, and each Q is a naturally occurring nucleobase or a non-naturally occurring nucleobase with the provisos defined in

claim 1.

4. A peptide nucleic acid probe according to anyone of claims 1 to 3, wherein Z is NH or O, and R^2 is H or the side chain of Ala, Asp, Cys, Glu, His, HomoCys, Lys, Orn, Ser or Thr, and Q is a nucleobase selected from thymine, adenine, cytosine, guanine, uracil, iso-C, iso-G and 2,6-diaminopurine with the provisos defined in claim 1.

5. A peptide nucleic acid probe according to anyone of claims 1 to 4 of formula (V)



(V)

wherein R^4 is H or the side chain of Ala, Asp, Cys, Glu, His, HomoCys, Lys, Orn, Ser or Thr, and Q is as defined in claim 4 with the provisos defined in claim 1.

6. A peptide nucleic acid probe according to anyone of claims 1 to 5 further comprising one or more labels which may be mutually identical or different, and/or one or more linkers which may be mutually identical or different with the provisos defined in claim 1.

7. Method for detecting mycobacteria of the Mycobacterium tuberculosis Complex in a sample comprising

(1) contacting any rRNA optionally present in said sample with one or more peptide nucleic acid probes according to anyone of claims 1 to 6 under conditions, whereby hybrids between said probe(s) and said rRNA are formed, and

(2) observing or measuring said hybridisation, and relating said observation or measurement to the presence of mycobacteria of the Mycobacterium tuberculosis Complex in said sample.

8. Method according to claim 7,
c h a r a c t e r i s e d in that the hybrids are captured on a solid phase before measuring the extent of hybridisation.

9. Method according to claim 7,
c h a r a c t e r i s e d in that a peptide nucleic acid probe according to anyone of claims 1 to 6 are used for capturing the hybrids.

10. A method according to anyone of claims 7 to 9,
c h a r a c t e r i s e d in that a signal amplifying system is used for measuring the resulting hybridisation.

11. Kit for detecting mycobacteria of the Mycobacterium tuberculosis Complex,
c h a r a c t e r i s e d in that said kit comprises at least one peptide nucleic acid probe according to anyone of claims 1 to 6, and a detection system with at least one detecting reagent.

12. Kit according to claim 11,
c h a r a c t e r i s e d in that it further comprises a solid phase capture system.

ABSTRACT

NOVEL PROBES FOR THE DETECTION OF MYCOBACTERIA OF THE MYCOBACTERIUM TUBERCULOSIS COMPLEX

Novel hybridisation assay probes for detecting mycobacteria of the Mycobacterium tuberculosis Complex (MTC) are provided. The probes detect 16S rRNA of MTC. Such probes are capable of detecting the organisms in test samples, e.g. expectorates, sputum, aspirates, urine, blood and tissue sections, food, soil and water.

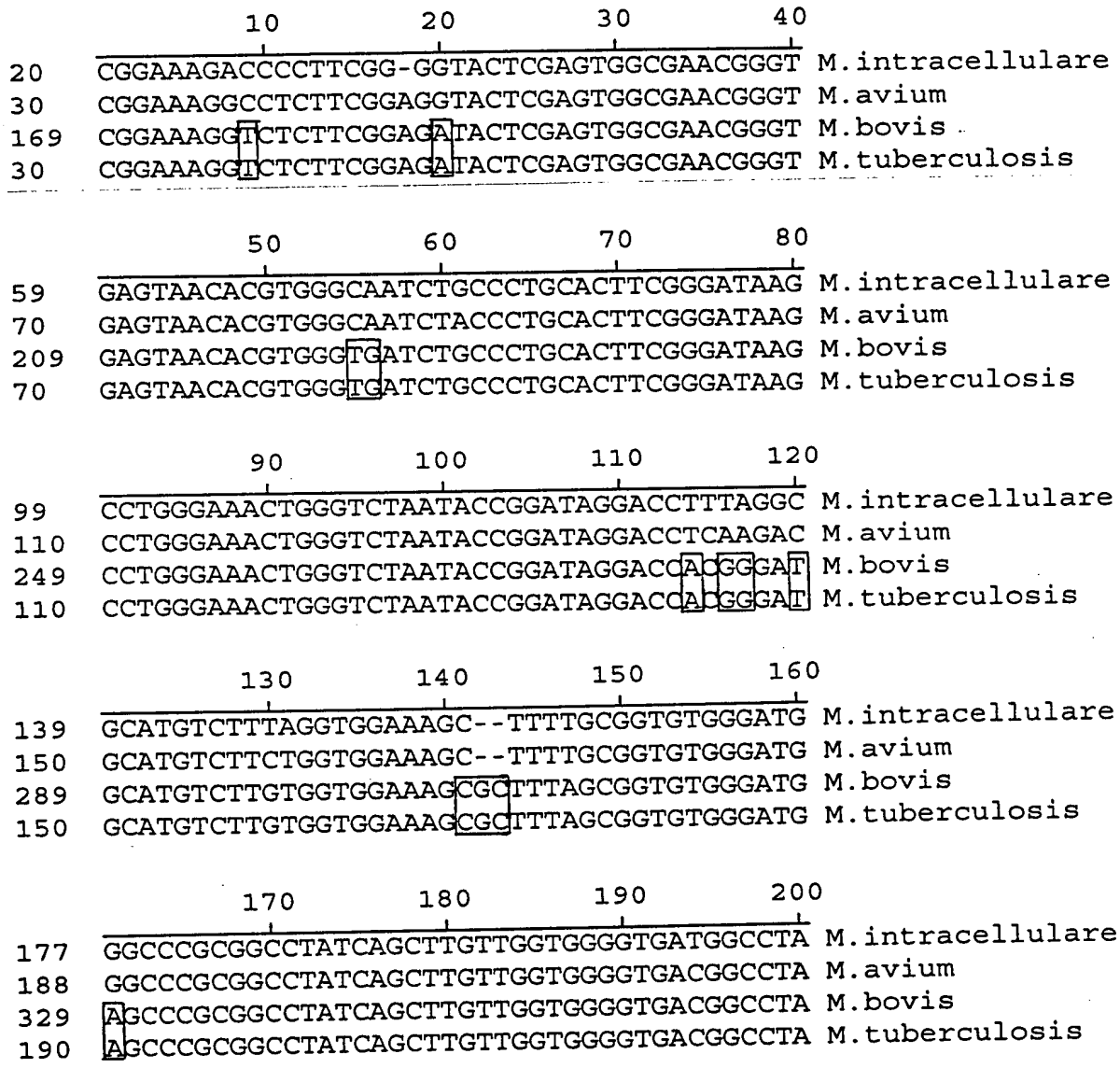

10 20 30 40
20 CGGAAAGACCCCTTCGG-GGTACTCGAGTGGCGAACGGGT M.intracellulare
30 CGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAACGGGT M.avium
169 CGGAAAGGTCTCTTCGGAGATACTCGAGTGGCGAACGGGT M.bovis
30 CGGAAAGGTCTCTTCGGAGATACTCGAGTGGCGAACGGGT M.tuberculosis
50 60 70 80
59 GAGTAACACGTGGGCAATCTGCCCTGCACTTCGGGATAAG M.intracellulare
70 GAGTAACACGTGGGCAATCTACCCTGCACTTCGGGATAAG M.avium
209 GAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAAG M.bovis
70 GAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAAG M.tuberculosis
90 100 110 120
99 CCTGGGAAACTGGGTCTAATACCGGATAGGACCTTTAGGC M.intracellulare
110 CCTGGGAAACTGGGTCTAATACCGGATAGGACCTCAAGAC M.avium
249 CCTGGGAAACTGGGTCTAATACCGGATAGGACCACGGGAT M.bovis
110 CCTGGGAAACTGGGTCTAATACCGGATAGGACCACGGGAT M.tuberculosis
130 140 150 160
139 GCATGTCTTTAGGTGGAAAGC--TTTTGCGGTGTGGGATG M.intracellulare
150 GCATGTCTTCTGGTGGAAAGC--TTTTGCGGTGTGGGATG M.avium
289 GCATGTCTTGTGGTGGAAAGCGCTTTAGCGGTGTGGGATG M.bovis
150 GCATGTCTTGTGGTGGAAAGCGCTTTAGCGGTGTGGGATG M.tuberculosis
170 180 190 200
177 GGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTA M.intracellulare
188 GGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTA M.avium
329 AGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTA M.bovis
190 AGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTA M.tuberculosis

Figure 1

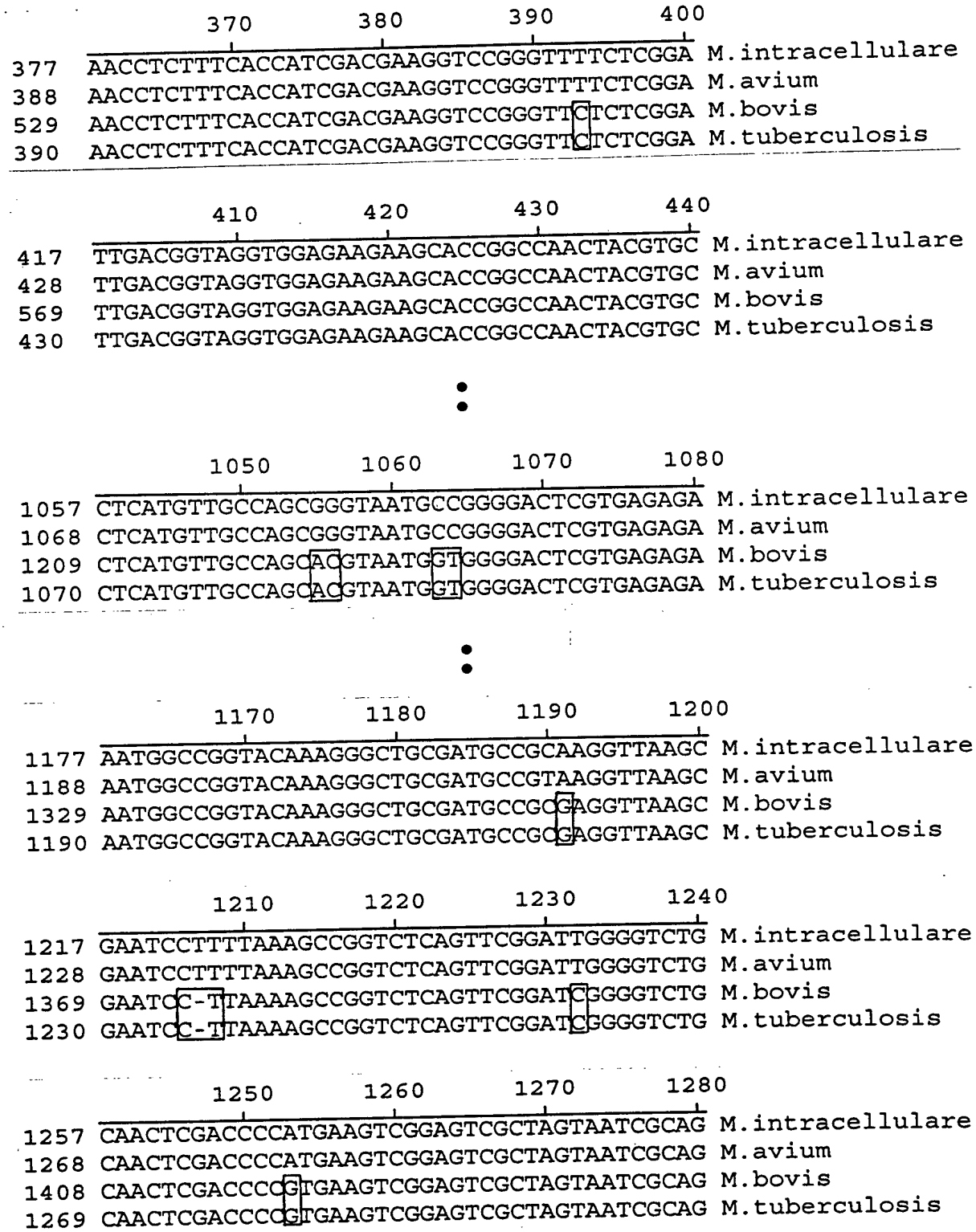

370 380 390 400
377 AACCTCTTTCACCATCGACGAAGGTCCGGGTTTTCTCGGA M.intracellulare
388 AACCTCTTTCACCATCGACGAAGGTCCGGGTTTTCTCGGA M.avium
529 AACCTCTTTCACCATCGACGAAGGTCCGGGTTCTCTCGGA M.bovis
390 AACCTCTTTCACCATCGACGAAGGTCCGGGTTCTCTCGGA M.tuberculosis
410 420 430 440
417 TTGACGGTAGGTGGAGAAGAAGCACCGGCCAACTACGTGC M.intracellulare
428 TTGACGGTAGGTGGAGAAGAAGCACCGGCCAACTACGTGC M.avium
569 TTGACGGTAGGTGGAGAAGAAGCACCGGCCAACTACGTGC M.bovis
430 TTGACGGTAGGTGGAGAAGAAGCACCGGCCAACTACGTGC M.tuberculosis
1050 1060 1070 1080
1057 CTCATGTTGCCAGCGGGTAATGCCGGGGACTCGTGAGAGA M.intracellulare
1068 CTCATGTTGCCAGCGGGTAATGCCGGGGACTCGTGAGAGA M.avium
1209 CTCATGTTGCCAGCACGTAATGGTGGGGACTCGTGAGAGA M.bovis
1070 CTCATGTTGCCAGCACGTAATGGTGGGGACTCGTGAGAGA M.tuberculosis
1170 1180 1190 1200
1177 AATGGCCGGTACAAAGGGCTGCGATGCCGCAAGGTTAAGC M.intracellulare
1188 AATGGCCGGTACAAAGGGCTGCGATGCCGTAAGGTTAAGC M.avium
1329 AATGGCCGGTACAAAGGGCTGCGATGCCGCGAGGTTAAGC M.bovis
1190 AATGGCCGGTACAAAGGGCTGCGATGCCGCGAGGTTAAGC M.tuberculosis
1210 1220 1230 1240
1217 GAATCCTTTTAAAGCCGGTCTCAGTTCGGATTGGGGTCTG M.intracellulare
1228 GAATCCTTTTAAAGCCGGTCTCAGTTCGGATTGGGGTCTG M.avium
1369 GAATCC-TTAAAAGCCGGTCTCAGTTCGGATCGGGGTCTG M.bovis
1230 GAATCC-TTAAAAGCCGGTCTCAGTTCGGATCGGGGTCTG M.tuberculosis
1250 1260 1270 1280
1257 CAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAG M.intracellulare
1268 CAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAG M.avium
1408 CAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAG M.bovis
1269 CAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAG M.tuberculosis

Figure 2